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ANIMAL AND PLANT HEALTH INSPECTION SERVICE
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SAM - 113

9 CFR 113.144	Revised Aug. 19, 1983	Parainfluenza-3
Standard Requirement	Supersedes June 7, 1971	Agent

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF PARAINFLUENZA-3 NEUTRALIZING ANTIBODY

(Constant Virus - Varying Serum Method)

A. SUMMARY

This is an in vitro assay method which employs a cell culture system for determining the antibody titer of serum against Parainfluenza-3 (PI-3) virus.

B. MATERIALS

1. Cell Cultures

Multiple 24-well disposable plates (16 mm well diameter) are needed (.5 ml/well) with primary bovine embryonic kidney (EBK) cells, 2nd through 5th passage. Cells must be free of extraneous agents. The cells are seeded at a density that will produce 80% confluency after 1 day of incubation.

a. Growth Medium

The cells are grown in Minimum Essential Medium (MEM) with 10% fetal calf serum and additives (Appendix 1) at a temperature of 35 to 37 C in an incubator containing an atmosphere of 5% carbon dioxide (CO_2) and a relative humidity of 70 to 80%. Growth medium is not changed unless excess acidity occurs or cells are not growing well.

b. Diluent

Maintenance media (Appendix 2) without serum is used to make dilutions of the serum and virus.

c. Indicator Virus

National Veterinary Services Laboratories (NVSL) reference PI-3 virus is used.

2. Guinea Pig Red Blood Cells (RBC) for the Hemadsorption (HAd) Test

a. Blood from healthy guinea pigs is collected aseptically in an equal volume of sterile Alsever's solution (Appendix 3).

b. The RBC are washed 3 times in Alsever's solution and sedimented each time by centrifugation at 1,000 rpm (250 X g) for 15 minutes.

c. For the HAd test, the RBC are diluted to a 0.5% suspension in phosphate buffered saline (PBS) (Appendix 4).

C. METHOD

1. Dilution of Indicator Virus

The indicator virus is diluted to contain 100 to 500 $TCID_{50}$/0.1 ml, and 0.1 ml is inoculated into each tissue culture well in the test system. This dilution is determined by previous titrations and is designated as the "stock virus".

Calculation of the dilution factor is as follows:

Divide the titer of the indicator virus by the desired titer of stock virus. This equals the dilution factor. Example:

$$\frac{\textit{Indicator virus titer}}{\textit{Stock virus titer wanted}} = \textit{dilution factor}$$

$$\frac{1{,}000{,}000\ TCID_{50}/0.1\ ml}{200\ TCID_{50}/0.1\ ml} = 5{,}000$$

The indicator virus is diluted 1:5,000.

2. Dilution of Test Serum

The serum is heat-inactivated at 56 C for 30 minutes. Serial 2-fold dilutions are made in sterile tubes containing diluent. Transfers are made with a 1 ml pipette and mixing is done by using a mixer (Vortex or similar type, no endorsement expressed or implied). Two-fold dilutions are made as follows:

a. One ml diluent is added to tubes 2, 3, 4, and 5.

b. One ml serum is added to tubes 1 and 2. Pipette is discarded and tube 2 is mixed. Tube 1 contains 1 ml of undiluted serum. Tube 2 contains a 1:2 dilution of the serum.

c. One ml is transferred from tube 2 to tube 3. Pipette is discarded and tube 3 is mixed. Tube 3 contains a 1:4 dilution of the serum.

d. This process is continued until the desired number of serum dilutions are made. One ml from the last serum dilution tube is discarded.

3. Serum Neutralization of Virus

An equal volume of stock virus (1 ml) is added to each serum dilution tube (1 ml), mixed, and allowed to incubate at room temperature for 45 minutes. Each of 5 cell culture wells are inoculated with 0.2 ml of the serum-virus mixture. The mixing of equal volumes of serum and virus results in a further 2-fold dilution of serum. Thus, the undiluted serum (tube 1) becomes a 1:2 final dilution, the initial 1:2 becomes 1:4, etc.

4. Controls

a. The stock virus is titrated by preparing serial 10-fold dilutions (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and allowing them to remain at room temperature along with the serum-virus mixtures. Five cell culture wells are inoculated with 0.1 ml of each virus dilution.

b. A known negative serum control is tested at a 1:2 dilution along with the test serums.

c. Five uninoculated cell culture wells are incubated and processed

along with the other cultures as a check on the test system.

5. Interpretation

The 50% endpoints of the serum and virus are calculated by the method of Reed and Muench or Spearman-Karber.

The stock virus 50% endpoint must be between 100 and 500 $TCID_{50}$/0.1 ml for a test to be valid. The negative serum control must not neutralize virus. The cells in the uninoculated control tubes must remain normal.

6. Incubation and Reading of Tests for Parainfluenza-3

The inoculated EBK 24-well plates are incubated at 35 to 37 C for 4 to 6 days. The cell layers are then examined by one or both of the following methods:

a. Cytopathic Effect (CPE):

The wells are examined for CPE typical of PI-3 virus. The number of wells found positive and negative for CPE are recorded and the 50% endpoints calculated.

b. Hemadsorption (HAd) Test:

(1) Fluids are poured from the plates.

(2) The cells are washed once with PBS (Appendix 4).

(3) To each well is added 1 ml of a 0.5% suspension of RBC.

(4) The plates are allowed to stand 15 to 20 minutes at room temperature.

(5) The suspension of RBC is poured off and the monolayers are

washed 3 times with PBS.

(6) The PBS is drained from the plates and the monolayers are examined microscopically for hemadsorption.

The number of wells positive and negative for HAd are recorded and the 50% endpoints calculated.

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APPENDIX

1. Growth Medium

Edamin	.5	%
MEM (Eagle with Earles' salts) q.s. ad	100.0	%
Antibiotics - Penicillin	100.0	units/ml
Streptomycin	100.0	mcg/ml
Gentamicin	50.0	mcg/ml
Amphotericn B	2.5	mcg/ml
Add 10% fetal calf serum		
L-Glutamine	1.0	%

2. Maintenance Medium

Edamin	0.5	%
MEM (Eagles with Earles' salts) q.s. ad	100.0	%
Antibiotics - Penicillin	100.0	units/ml
Streptomycin	100.0	mcg/ml
Gentamicin	30.0	mcg/ml
Amphotericin B	2.5	mcg/ml

3. Alsever's Solution

Dextrose	2.05 %
Sodium citrate	0.8 %
Sodium chloride	0.42 %
Citric acid	0.055%
Distilled H_2O q.s. ad	100.0 %

4. Phosphate Buffered Saline (PBS-Dulbecco)

NaC1	0.8 %
KC1	0.02 %
Na_2HPO_4	0.115%
KH_2PO_4	0.02 %
$CaC1_2$ (anhy.)	0.01 %
$MgC1_2$ $6H_2O$	0.01 %
Distilled H_2O q.s. ad	100.0 %